Small-Molecule Anthracene-Induced Cytotoxicity and Induction of Apoptosis through Generation of Reactive Oxygen Species

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A series of anthracene derivatives have been synthesized, and their potential individual cytotoxicity was evaluated using Jurkat T cells and peripheral blood mononuclear cells (PBMCs) *in vitro*. These compounds, except for 2l, showed less cytotoxicity in PBMCs than mitoxantrone. We also analyzed the antiproliferative activity of these derivatives using the annexin V/propidium iodide assay. These synthetic compounds induced apoptosis, thus leading to antitumor effects. Compounds 2b, 2e, 2f, 2g, 2h, 2i, 2j, and mitoxantrone produced dose-dependent cytotoxicity, while the antiproliferative activity of the anthracene pharmacophore was retained in Jurkat T cells base on the detection of DNA degradation and membrane unpacking. These clearly indicate a correlation between cytotoxicity and antitumor activity. Unlike mitoxantrone, cytotoxic properties were observed, as documented by the reactivity of these novel compounds against Jurkat T cells and PBMCs as normal cells, respectively. Various concentrations of 2b, 2e, 2f, 2g, 2h, 2i, and 2j preparations also inhibited Jurkat T cell proliferation and induced apoptosis of Jurkat T cells, potentially confirmed through the detection of DNA degradation and membrane unpacking. In the present report we also investigated the antiinflammatory activity against phorbol-12-myristate-13-acetate induced superoxide anion production, a marker for an inflammatory mediator produced by neutrophils, with IC_{50} (μM) values of 2b, 2h, 2l, and 2o of 4.28±0.89, 3.31±0.88, 4.38±0.25, and 5.45±1.78, respectively. These results suggest that, in addition to the specific chromosomal aberrations and cell death, elevated apoptosis could also be a marker for exposure to anthracene derivatives.

Key words anthracene; Jurkat T cell; peripheral blood mononuclear cell; apoptosis; flow cytometry; propidium iodide

The induction of apoptosis is considered to be ideal method to eliminate cancer cells, and apoptosis-inducing agents are viewed as potential antitumor agents.[1] Apoptosis is involved in many normal biological processes, such as embryonic and T cell development, metamorphosis, and hormone-dependent atrophy; it can also be induced by a variety of cytotoxic processes.[2] Laboratory evidence suggests that many anticancer agents exert their effect by altering the ratio between apoptosis and cellular proliferation.[3] Anthracyclines interfere with topoisomerase II, intercalate DNA, and are substrates for P glycoprotein and multidrug resistance-associated protein.[4] 7,12-Dimethylbenz[a]anthracene (DMBA) induces apoptosis of mammary cancer such as leukemia in Long–Evans (LE) rats.[5] We have previously reported the synthesis and cytotoxicity evaluations of a series of anthracenes and anthraquinones in cultured murine and human tumor cells and their biological evaluation.[6—12] Although most the compounds have less antiproliferative activity than mitoxantrone (**1a**), some were found to have significantly activity against different cancer cell lines and activation of human telomerase reverse-transcriptase expression. Some of the small-molecule anthracene derivatives inhibit proliferation, induce apoptosis in cancer cells, and retard tumor growth. Thus they have potential for development as clinical agents for the treatment of advanced cancer and other chemoresistant tumors, although their mechanism of action is still uncertain.

Part of the difficulty in this research may lie in the historical preference for carrying out cytotoxic screening regimens using *in vivo* or *in vitro* assays. To determine cytotoxicity and to understand the mechanism required for their activity, this study investigated the antiproliferative and cytotoxic potency of a set of structurally diverse anthracene derivatives. Furthermore, because the antitumor potency of the structurally similar anthracenes differs substantially between peripheral blood mononuclear cells (PBMCs) and Jurkat T cell lines, we have investigated the rationale for this biological behavior by carrying out a series of biochemical and cellular studies. The cytotoxicity mechanism consists of the induction of apoptosis, whereas the selectivity against tumor cells is founded on a specific dose dependent pathway of drug incorporation. The tumor microenvironment plays a critical role in determining the fate of tumor cells. Therefore it represents a promising target for small-molecule inhibitors to shift the apoptotic threshold in cancer cells after treatment with standard chemotherapy. Thus the search for new chromophore-modified molecules with antiproliferative properties is a very active area of research. Early studies on the geometry of binding of intercalators to DNA relied on viscometry, gel electrophoresis, and circular and electric dichroism to determine the lengthening, stiffening, and unwinding of the helix which accompany intercalation and the orientation of the chromophore.[13] Therefore our efforts in this area were directed toward modification of substitution on anthracene as potent cytotoxic analogues. In this study, our continuing investigation of structure–activity relationships (SARs) of the anthracene class of antitumor agents, we further investigated the biological properties of 9-alkoxy-substituted congeners of anthracene. The most active compounds displaying *in vitro* cytotoxicity in Jurkat T cells were **2b**, **2e**, **2f**

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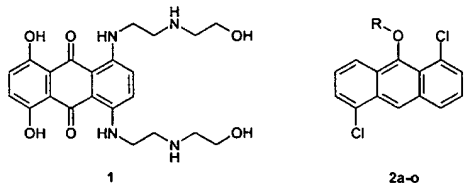

1 2a-o

Chart 1. Structures of Anthraquinone and Anthracene: Mitoxantrone and 9-Alkoxy 1,5-Dichloroanthracenes

and **2j**. Mechanistic studies performed with these compounds showed marked differences to mitoxantrone. This newly uncovered activity of the novel anthracenes suggests antitumor potential with respect to disturbance of tumor cell differentiation, in addition to induction of apoptotic death in Jurkat T cells. In this report, we describe a useful flow cytometric technique to evaluate the apoptotic properties of some selected small molecules on living cells using fluorescent probes. The probes include those derivatives considered to be specific for Jurkat T cells, as well as mitoxantrone, which is considered to be more specific for living cells, and other fluorescent probes.[14] After mitoxantrone treatment, Jurkat T cells also display an increased propensity for apoptosis. Thus, in addition to their cytotoxicity as a measure of their antiproliferative activity against the growth of Jurkat T cells and PBMCs, we also evaluated their apoptotic properties using a flow cytometric assay. Apoptosis by mitoxantrone and anthracene derivatives was subsequently observed in other lines derived from tumors and their metastases. We report here the characterization and anticancer activity of mitoxantrone and anthracene derivatives, which can be prototypes for new anthracene apoptotic agents with potential for cancer treatment in terms of their membrane damaging effects and in a dose-dependent manner.

MATERIALS AND METHODS

Chemicals The synthesis and subsequent analysis of anthracenes were described previously.[6]

Culture of Human PBMCs and Jurkat T Cells[15,16] Human PBMCs enriched from whole blood were isolated by density gradient centrifugation and cultured in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, U.S.A.), supplemented with penicillin, streptomycin, L-glutamine (Bio Whittaker, Walkersville, MD, U.S.A.), and 10% heat-inactivated human AB serum (Sigma Chemical Co., St. Louis, MO, U.S.A.). Human leukemia T lymphocytes (Jurkat T cells) were obtained from Hayashibara Biochemical Laboratories Inc. (Department of Anesthesiology, National Defense Medical Center). Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin plus streptomycin (Gibco BRL) in an atmosphere of air supplemented with 5% CO_2 at 37 °C. The cells were seeded every 2 or 3 d. Cells were grown in a CO_2 incubator containing 5% CO_2 at 37 °C, at a concentration of 10^6 cells/ml. Aliquots containing 1—2×10^6 cells/ml were used for all experiments.

***In Vitro* Cytotoxicity Evaluation**[17] Cytotoxicity studies were performed in triplicate in the presence of increasing drug concentrations in 24 plates. The compounds were evaluated for their potential toxic effects on human PBMCs and Jurkat T cells. PBMCs and Jurkat T cells were washed extensively and suspended at a density of 1×10^6 cells/ml. To assess the *in vitro* cytotoxicity, approximately 1×10^6 cells were seeded in the wells of 24-well plates at varying concentrations (7.5, 15, 30, 75, 150, and 300 μM) of test compounds dissolved in DMSO in triplicate. After cell density and viability determination (trypan blue dye-exclusion method), cells were distributed into 24-well tissue culture plates to which diluted compound solutions and medium had been added. After 24 h of continuous exposure to the drug, the cell concentration was determined with a Coulter counter. Growth inhibition was calculated for each drug concentration using the following formula:

$$\%\ \text{growth inhibition} = [1 - (\text{cell number treated}/\text{cell number DMSO alone})] \times 100$$

Cell Preparation and Test Compound Pretreatment Test compound pretreatment was performed 24 h prior to apoptosis induction as follows: Jurkat cells were suspended in a fresh medium at 1×10^6 cells/ml, then 7.5, 75, 150, and 300 μM of compounds **2b**, **2e**, **2f**, **2g**, **2h**, **2i**, and **2j** were added and incubated for 10 min at 37 °C, respectively. As test compounds rapidly reacted with cells and medium components, virtually all test compounds disappeared during the 10-min incubation period. The treated cells were separated from the medium by centrifugation at 500×***g*** for 5 min, resuspended in fresh medium at 0.5×10^6 cells/ml, and cultured for 24 h. In addition to compounds **2b**, **2e**, **2f**, **2g**, **2h**, **2i**, **2j**, and mitoxantrone, the effects of various derivatives were also studied using the same procedure.[17,18]

Flow Cytometry Analysis in Jurkat T Cells (DNA Fragmentation Assay) To confirm that the DNA loss induced by test compounds was due to apoptosis, Jurkat T cells were cultured with varying concentrations of compounds for 24 h and stained with propidium iodide (PI) solution (50 μg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100).[18] Apoptotic nuclei produce a broad hypodiploid DNA peak, which is easily distinguished from the narrow peak of cells with normal DNA content.[19] In contrast to the cells cultured in control medium, large hypodiploid DNA peaks were seen for Jurkat cells as early as 2 h after exposure to test compounds. Cell survival, as assessed by clonogenic assay and flow cytometry, was also significantly increased in cells with high apoptotic ratios. Cell death was assayed via flow cytometry (Becton Dickinson FACSan) using PI staining. Flow cytometry analysis of test compounds induced apoptosis in Jurkat T cells (Figs. 1, 2). Control and test compound-pretreated cells were collected and resuspended in fresh medium at 1×10^6 cells/ml. Apoptosis-inducing reagents (**2b**, **2e**, **2f**, **2g**, **2h**, **2i**, **2j**, and mitoxantrone) were added at the following final concentrations: 7.5, 75, 150, and 300 μM, respectively. Subsequent to the addition of each chemical, the cells were incubated for the indicated times in a CO_2 incubator at 37 °C with 5% CO_2. Thus exposure of Jurkat T cells to test compounds resulted in morphologic alterations such as chromatin condensation and membrane asymmetry which are associated with apoptosis.[20-22] Results were tabulated for the indicated number of experimental samples. Group means were compared using Student's *t*-test for unpaired or paired samples with a two-tailed distribution. Cells were lysed in a hypotonic buffer containing 0.1% Triton X-100 and 200 mg/ml of Rnase A,

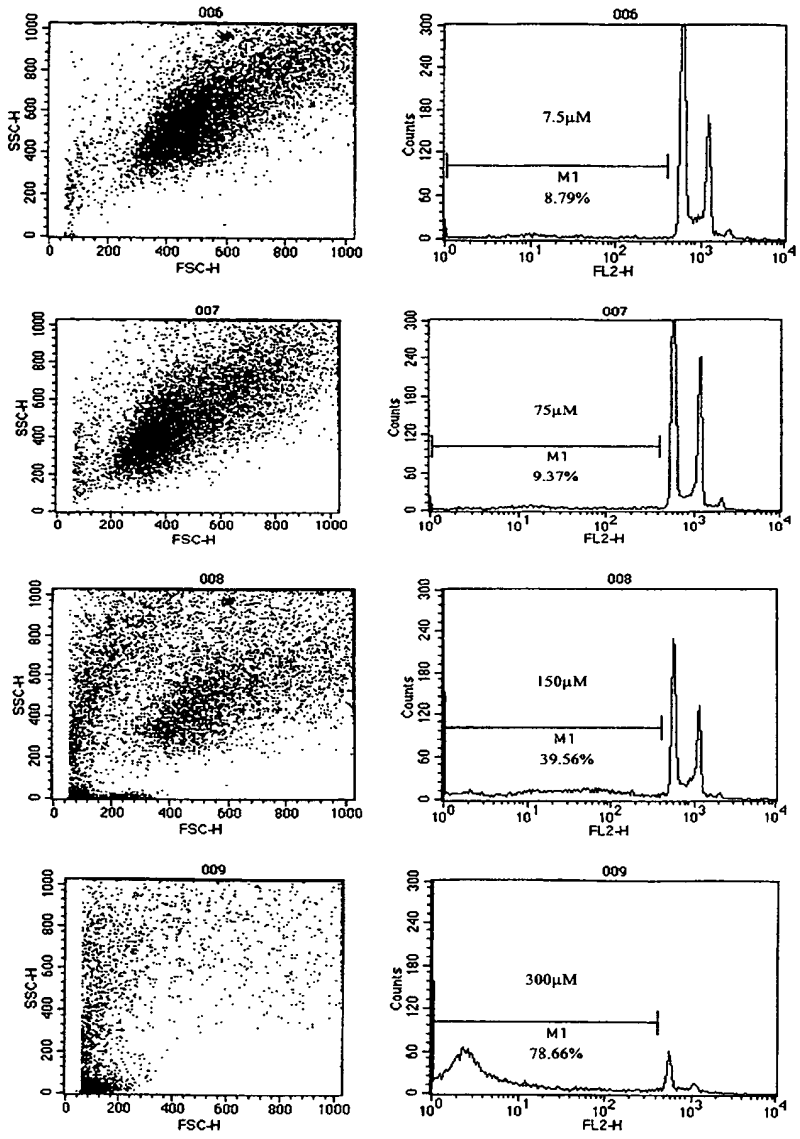

Fig. 1. Flow Cytometric Histogram Analysis of Compound **2h**-Induced Apoptosis of Jurkat T Cells at 7.5, 75, 150, and 300 μM

The cells were stained with PI after incubation in medium. The results are representative of at least three independent experiments, with approximately 10000 live cells analyzed per experiment.

stained with PI, and analyzed with a flow cytometer. The PI area indicates the DNA amount. PI width was simultaneously measured to exclude duplicated events, in which two or more cells were counted as a single event by mistake, and the results are representative of at least four independent experiments. Approximately 10000 live cells were analyzed per experiment.

Human Neutrophil Isolation For the preparation of human leukocytes, all experimental protocols were approved by our Institutional Review Board in accordance with international guidelines. Preparation of human neutrophils was obtained by venopuncture from healthy adult volunteers and collected into syringes containing heparin (20 U/ml blood) according to our previous report.[23] Neutrophils were isolated using the Ficoll gradient centrifugation method, followed by lysis of contaminating erythrocytes. Briefly, blood samples were mixed with an equal volume of 3% dextran solution in a 50-ml centrifuge tube and incubated in an upright position for 30—40 min at room temperature to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was then collected and subjected to centrifugation at 250×***g*** for 15 min at 4 °C. After centrifugation, the pellet was suspended immediately in a volume of phosphate-buffered saline (PBS) equal to the starting volume of blood. The cell suspension was then apportioned at 6 ml per tube into 15-ml centrifuge tubes, followed by laying 8 ml of 1.077 g/ml Ficoll solution (Histopaque 1077; Sigma Chemical) beneath the cell suspension using a pipette. After centrifugation at 400×***g*** for 40 min at 20 °C without a break, the upper (PBS) and lower (Ficoll) layers were carefully removed, leaving the granulocyte/erythrocyte pellet. To remove residual erythrocytes, the pellet was suspended in 10 ml of cold lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediaminetetraacetate (EDTA), pH 7.4. The remaining neu-

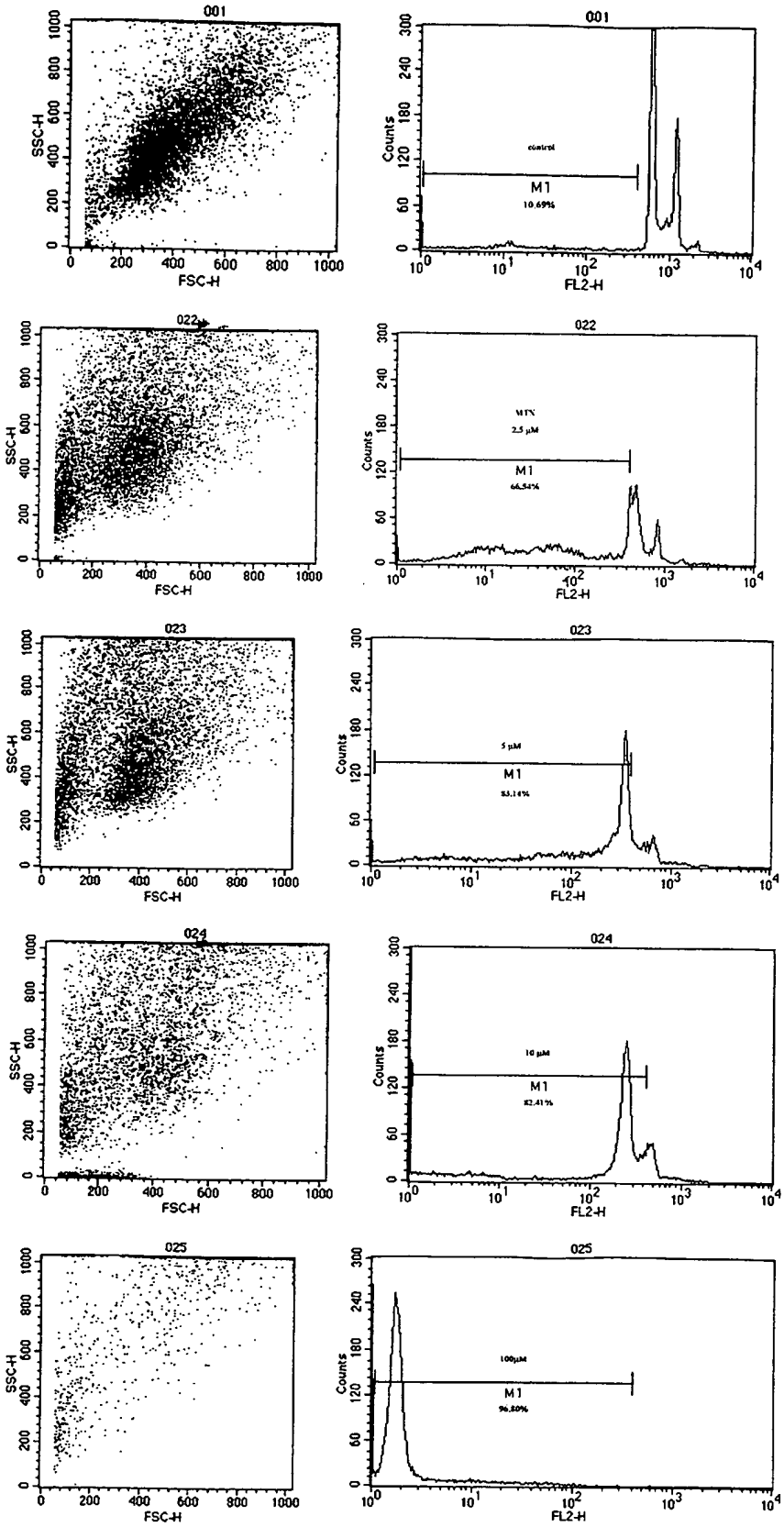

Fig. 2. Flow Cytometric Histogram Analysis of Mitoxantrone-Induced Apoptosis of Jurkat T Cells at 2.5, 5, 10, and 100 μM and Controls

The cells were stained with PI after incubation in medium. The results are representative of at least three independent experiments, with approximately 10000 live cells analyzed per experiment.

trophils were then pelleted, washed twice with ice-cold PBS, and suspended in an adequate volume of ice cold Hanks' buffered saline solution (HBSS) until further manipulation. The preparation contained more than 95% neutrophils, as estimated by counting 200 cells under a microscope after Giemsa (Sigma Chemical) staining. In all cases, except where indicated, neutrophils were pretreated with test compounds at concentrations ranging from 1 to 50 μg/ml in HBSS for 10 min at 37 °C.

Measurement of Extracellular Generation of Superoxide Anion ($O_2^{\cdot-}$) Extracellular $O_2^{\cdot-}$ generation was evaluated as in our previous report.[23] Phorbol myristate acetate (PMA) (2.5 μg/ml)-induced production of $O_2^{\cdot-}$ by leukocytes was determined in the presence or absence of 1 to 50 μM of test compounds based on superoxide dismutase-inhibitable cytochrome c (80 μM) reduction by measuring the changes of absorbance at 550 nm (ΔOD_{550}) in the presence of superoxide dismutase (133 U/ml) at 37 °C for 1 h. Staurosporine, a protein kinase C-inhibitor, was included as a positive control for the abolishment of extracellular $O_2^{\cdot-}$ production. Data are expressed as $O_2^{\cdot-}$ nmol/2×10^5 cells/h, using the molar extinction coefficient of 2.1×10^4 l/mol for cytochrome c, with a path length of 8 mm.

Estimation of Cell Viability Cell viability was determined according to our previously reported method[24] after incubation of cells (2×10^6/ml) with test drugs for 2 h in 5-ml polystyrene round-bottomed tubes (Falcon, Becton Dickinson). This method can be adapted for a flow cytometer by adding 10 μg/ml of PI, which is excluded by viable cells but which, when taken up by dead or dying cells, binds to nucleic acids and fluoresces red. The viable cells can be further identified by adding 100 ng/ml of fluorescein diacetate (FDA), which is not fluorescent and which is taken up by cells and converted to fluorescein by an intracellular esterase. The cell retains fluorescein if the plasma membrane is intact. After incubation with test compounds, cell suspensions were further incubated with PI and FDA at room temperature for 10 min and analyzed immediately on a flow cytometer (FACSCalibur™; Becton Dickinson) by recording forward and light scatter, and red (>630 nm) and green (520 nm) fluorescence. After gating for light scatter to include single cells and to exclude clumps and debris, cell populations were displayed by green (viable) *versus* red (dead) fluorescence. Cell viability (dead cell %) was calculated using CellQuest™ software (Becton Dickinson) on a Power Macintosh 7300/200 computer. Alternatively, cell viability was further compared using a cytotoxicity detection kit (Roche, Germany). This kit measures cytotoxicity and cell lysis by detecting lactate dehydrogenase (LDH) activity released from damaged cells.

Statistical Analysis All values in the text and figures are expressed as mean±S.E.M. Data were analyzed by one-way or two-way analysis of variance (ANOVA) depending on the number of experimental variables followed by the *post-hoc* Dunnett's *t*-test for multiple comparisons. Concentration dependence was analyzed by simple linear regression analysis of response levels against concentrations of compound and testing the slope of the regression line against 0 using Student's *t*-test. Values of $p<0.05$ were considered significant. The mean and standard deviation are designated by X±S.D. The probable level of significance ($p<0.01$) between test and control samples was determined using the Student's *t*-test with the raw data.

RESULTS

The small-molecule anthracene derivatives demonstrated potent cytotoxicity in the murine and human screening *in vitro*. It was previously found that **2c**, **2d**, and **2l** had greater cytotoxic activity than the other compounds in rat glioma C6 cell lines using the XTT colormetric assay.[6] However, analysis of the antiproliferative activity of these anthrancene derivatives did not reveal a correlation between the different cell lines. A similar observation was made for these compounds when flow cytometric assay results and cytotoxicity against PBMCs and Jurkat T cells were examined. Flow cytometry was used to measure the accumulation of test compounds and mitoxantrone in cells. The structures of the 9-alkoxyl-substituted anthracenes are listed in Table 1, together with relevant biological properties. To probe the importance of the anthracene structure on antiproliferative activity in primary cells, the antiproliferative activity in Jurkat T cells and PBMCs of a series of anthracenes was evaluated. As shown in Table 1, these compounds exhibited various levels of antiproliferative activity with no associated cytotoxicity. The biological assay procedures used were identical to those described below.

Jurkat T cells, a human acute T cell leukemia cell line, were pretreated with various concentrations (300, 150, 75, 7.5 μM) of test compounds. After 24 h, trypan blue dye was added to the pretreated compounds and control cells, and their cytotoxicity levels were measured. Anthracene derivatives, despite their higher electrophilicity, exhibited less cytotoxicity and antiproliferative activity than mitoxantrone. Thus no consistent relationship between the electronic effect on the aromatic side chain and cytotoxicity was observed. This suggests that the presence of the alkoxy group in the side chain accelerates the bioreductive activation leading to cell death. In our continuing investigation of the SARs of the anthracene class of antitumor agents, we further studied the biological properties of 9-substituted congeners of anthracene. Thus, in addition to the flow cytometric assay as a measure of apoptosis inhibition, we evaluated their antiproliferative activity against the growth of Jurkat T cells and PBMCs as normal cells and determined their potential to induce differentiation of these cell lines. *In vitro* assay in the Jurkat T cell line revealed that compounds that were O-linked by short alkyl chains (**2b**, **2e**, **2f**, **2g**, **2h**, **2i**, and **2j**) exhibited 2- to 18-fold increases in cytotoxic potency as compared with mitoxantrone. The weaker activity of compounds **2k**, **2l**, **2m**, and **2o** indicated that the elongation of alkyl chains and aromatic substitutions on the C-9 oxygen atom reduced the activity more than most alkyl chain derivatives in this system. The effects of mitoxantrone on the cell cycle, following exposure of Jurkat T cells to 2.5, 5, 10, or 100 μM for 24 h, were relatively dose dependent. It has been shown that at 24 h post exposure the percentage of viable cells was 66.54%, 73.14%, 82.41%, and 96.80%, respectively (Table 2). The flow cytometric histogram analysis for compounds **2b**, **2e**, **2f**, **2g**, **2h**, **2i**, and **2j** induced apoptosis of Jurkat T cells at 7.5, 75, 150, and 300 μM, as listed in Table 2.

Table 1. Cytotoxic Activity of 9-Alkoxy 1,5-Dichloroanthracene Derivatives on Jurkat T Cells and PBMCs

Compound	R	IC_{50} (μM)[a]	
		Jurkat T[b]	PBMCs[c]
2a	CH_3	51.2±3.1	75.0±5.8
2b	CH_2CH_3	2.2±0.2	65.3±7.5
2c	CH_2CH_2Cl	7.1±1.0	>75
2d	CH_2CH_2Br	9.2±1.1	>75
2e	$CH_2CH_2CH_3$	1.6±0.5	37.5±3.1
2f	$CH_2CH(CH_3)_2$	0.9±0.4	33.3±3.5
2g	$CH_2CH_2CH_2Cl$	3.4±0.8	19.0±1.5
2h	$CH_2CH_2CH_2CH_3$	0.4±0.1	35.8±3.1
2i	$CH_2CH_2CH(CH_3)_2$	0.6±0.5	38.5±2.6
2j	$CH_2CH_2CH_2CH_2Cl$	3.6±0.9	>75
2k	$(CH_2)_7CH_3$	48.5±3.5	>75
2l	$CH_2C_6H_5$	25.1±2.2	14.0±1.2
2m	$CH_2C_6H_4CH_3$(o)	49.9±3.7	>75
2n	$CH_2C_6H_4CH_3$(p)	4.1±2.1	>75
2o	$CH_2CH_2C_6H_5$	38.2±3.6	23.8±2.2
Mitoxantrone		0.2±0.1	16.8±1.5

a) IC_{50}, drug concentration inhibiting 50% of cellular growth following 48 h of drug exposure. Values are in μM and represent an average of three experiments. The variance for the IC_{50} was less than±20%. *b*) Jurkat T cells. *c*) PBMCs (human peripheral blood mononuclear cells).

Table 2. DNA Fragmentation Assay of Compounds (**2b**, **2e**, **2f**, **2g**, **2h**, **2i**, and **2j**) and Mitoxantrone on Flow Cytometry

Compound	R	Apoptosis (%)[a]			
		7.5 μM	75 μM	150 μM	300 μM
2b	CH_2CH_3	7.78	11.88	21.91	63.30
2e	$CH_2CH_2CH_3$	8.14	12.09	43.19	81.58
2f	$CH_2CH(CH_3)_2$	10.91	13.08	53.30	73.53
2g	$CH_2CH_2CH_2Cl$	11.90	13.88	30.60	50.17
2h	$CH_2CH_2CH_2CH_3$	8.79	9.37	39.56	78.66
2i	$CH_2CH_2CH(CH_3)_2$	8.33	12.21	60.15	69.85
2j	$CH_2CH_2CH_2CH_2Cl$	11.28	13.75	21.25	44.44
Mitoxantrone		66.54[b]	73.14[c]	82.41[d]	96.80[e]

a) Jurkat T cells were cultured with varying concentrations of compounds for 24 h and stained with PI. Results are expressed as the means±standard errors of triplicate wells, and are representative of three independent experiments. *b*–*e*) The effects of mitoxantrone on the cell cycle following exposure of Jurkat T cells to 2.5, 5, 10, or 100 μM for 24 h were relatively dose dependent. At 24 h post exposure, the percentage of viable cells was 66.54%, 73.14%, 82.41%, and 96.80%, respectively.

In the present study, after we elucidated the extracellular $O_2^{\cdot-}$ generation by human neutrophils, the anthracene analogues displayed potent antiinflammatory activity against superoxide anion production. As shown in Table 4, compounds **2b**, **2e**, and **2l** were more effective than other compounds in this assay, with reactive oxygen species (ROS) inhibition (%) levels ranging from 48.53 to 71.63. They were almost 10-fold more potent than **2a** and 5-fold more potent than mitoxantrone, indicating that the relative length of the alkyl group in the substitution was optimal for antioxidative activity in human neutrophils. Furthermore, these compounds also concentration dependently inhibited superoxide anion production with an inhibitory percentage greater than 50% at 100 μM.

Small-molecule anthracenes or anthraquinones are known to be cytotoxic chemicals due to the action of free radicals inducing oxidative stress.[25] These free radicals may attack DNA and produce different types of DNA lesions.[26] Therefore we examined the cytotoxic effect of these compounds in peripheral human neutrophils. Table 4 also summarizes the significant cytotoxic effect of these compounds at concentrations ranging between 2—100 μM. In positive controls of triton 0.1% or 0.01%, they permeated the cell membrane for induction of cytotoxicity and significantly reduced cell viability to 27% and 98%, respectively. On the other hand, these results indicate that anthracence derivatives are more potent than mitoxantrone compounds as antiinflammatory agents with no significant cytotoxicity in human neutrophils. ROS, including $O_2^{\cdot-}$ and H_2O_2, are formed after a single-electron reduction of the quinone moiety of anthracyclines by mitochondrial, nuclear, or NADPH-cytochrome P450 reductase, thus producing a semiquinone free radical.[27] Free radicals sometimes result in the side effects seen in tumor cells and cardiac myocyte membranes.[28]

Table 3. Superoxide Production and Cell Viability in Human Neutrophils

Compound	R	IC_{50} (μM) of apoptosis[a]
2b	CH_2CH_3	0.20
2e	$CH_2CH_2CH_3$	0.21
2f	$CH(CH_3)_2$	0.19
2g	$CH_2CH_2CH_2Cl$	0.16
2h	$CH_2CH_2CH_2CH_3$	0.22
2i	$CH_2CH_2CH(CH_3)_2$	0.20
2j	$CH_2CH_2CH_2CH_2Cl$	0.16
Mitoxantrone		2.14

a) Jurkat T cells were cultured with varying concentrations of compounds for 24 h and stained with PI. Results are expressed as the means±standard errors of triplicate wells and are representative of three independent experiments.

Table 4. DNA Fragmentation Assay of Compounds (**2b**, **2e**, **2f**, **2g**, **2h**, **2i**, and **2j**) and Mitoxantrone on Flow Cytometry.

Compound	R	ROS inhibition (%) at 100 μM[a]	Dead cells (%) at 100 μM[b]
2a	CH_3	8.31±4.12	5.20±1.89
2b	CH_2CH_3	48.53±4.63	1.90±0.91
2e[c]	$CH_2CH_2CH_3$	71.63±6.24	1.50±0.83
2f	$CH_2CH(CH_3)_2$	29.38±10.85	1.20±0.64
2h	$CH_2CH_2CH_2CH_3$	23.31±0.88	1.80±0.62
2l	$CH_2C_6H_5$	53.27±2.37	1.30±0.13
2o	$CH_2CH_2C_6H_5$	15.40±2.30	1.20±0.35
Mitoxantrone		14.57±7.61	3.50±0.56

a) Extracellular $O_2^{\cdot-}$ generation was evaluated in human neutrophils. PMA (2.5 μg/ml)-induced production of $O_2^{\cdot-}$ by leukocytes was determined in the presence or absence of 1 to 50 μM of test compounds (type I) by superoxide dismutase-inhibitable cytochrome c (80 μM) reduction. Data are expressed as 50% inhibitory concentration (IC_{50}) or inhibition (%) at 50 μM. Values represent the mean±S.E.M. of 3—6 experiments. *b*) Cytotoxic effect was measured using flow cytometry by adding PI, which taken up by dead or dying cells binds to nucleic acids and fluoresces red. Values represent the mean±S.E.M. of 3 experiments. Dead cell % in vehicle control (0.25% DMSO), triton (0.01%), or emodin (100 μM) was 5.0±0.5, 27.3±3.6, or 0.1±1.2, respectively. *c*) IC_{50} of compound **2e** is 2.74±0.92 μM.

DISCUSSION AND CONCLUSIONS

Recently, it has been suggested that oxidative stress plays a role as a common mediator of apoptosis.[29,30] A number of naturally occurring products have been shown to have inhibitory effects on the proliferation of tumor cells associated with cell cycle blockage and apoptosis.[1] Neutrophils are programmed to undergo apoptosis after 24 h in peripheral blood,[31] so there is no need for an exogenous trigger for programmed cell death, since they are mostly short-lived.[32]

Since these neutrophils are short lived, in the absence of inflammation resting neutrophils undergo apoptosis in the circulation after 6—9 h.[33] Conversely, when neutrophils reach an inflammation site, apoptosis is delayed by inflammatory cytokines in the tissues, providing additional time for completion of the microbicidal function of neutrophil's.[34] To facilitate removal of ROS during PMA-induced oxidative stress and apoptosis, we chose to use the Jurkat T cell line assay. Flow cytometry has been extensively used to follow the apoptotic cascade and to enumerate apoptotic cells, both in cell cultures and, to a lesser extent, in tissue biopsies.[35] Apoptosis is also known to play an essential role as a protective mechanism against neoplastic development by eliminating damaged or abnormal cells.[36] The induction of apoptosis in cancer cells may represent a unifying concept for the mechanism of cancer chemoprevention, because aberrant proliferation and modulated apoptosis represent crucial early events in a multistep carcinogenic process.[37] The anthracycline antibiotic daunorubicin can induce programmed cell death (apoptosis) in cells.[38] Mitoxantrone induces apoptosis in Jurkat T cells, although the method by which it initiates this process remains to be completely defined. The mechanisms for small-molecule anthracene-, anthracenone- and anthraquinone-induced apoptosis have not yet been fully defined. However, the mechanism by which anthracycline-type drugs kill cells has been proposed to involve intercalation of the planar aglycone moiety into the DNA (affecting DNA replication and transcription) and redox cycling, resulting in the oxidative damage of cellular macromolecules and lipid membranes.[39] However, given that mitoxantrone causes minimal oxidative stress in target cells,[40,41] its apoptotic effects can be exclusively correlated with its ability to induce DNA damage, specifically DNA double-strand breaks, by direct interaction with a family of enzymes known as type II topoisomerases.[42] Topoisomerase II is a target for a number of chemotherapeutic agents used in the treatment of cancer. Its essential physiological role in modifying the topology of DNA involves the generation of transient double-strand breaks. Anticancer drugs, such as mitoxantrone, that target this enzyme interrupt its catalytic cycle and give rise to persistent double-strand breaks, which may be lethal to a cell.[43] A range of cytotoxic drugs, including many used in cancer therapy to induce cell death, has also been found to involve apoptosis.[14] Apoptosis is defined as programmed cell death that is a type of single-cell death without inflammation. Apoptosis is characterized morphologically by condensation of nuclear chromatin, compaction of cytoplasmic organelles, cell shrinkage, collapse of the mitochondrial membrane potential, and changes at the cell surface.[19] Apoptotic cells *in vivo* are rapidly phagocytosed, whereas rupture of the plasma membrane occurs only at a late stage *in vitro*. Usually apoptosis is accompanied by fragmentation of DNA into oligonucleosomal fragments with lengths that are multiples of 180—200 bp.[44] In contrast, the hallmark of necrosis is uncontrolled swelling followed by rupture of the plasma membrane.[20] DNA degradation tends to be non specific. The amount of DNA extracted from the cells, and hence the position of the apoptotic peak in the DNA histogram, depends on the type of cell under study. The DNA area may reveal apoptotic cells (see Fig. 1), which should be observed as a distinct peak. Necrotic cells, of which the DNA is degraded randomly, will have a reduced DNA content and distributed across the same region of the histogram. The cells in this study were stained with PI after incubation in medium. The results are representative of at least three independent experiments, with approximately 10000 live cells analyzed per experiment. The present results showed that nucleotides polymers are detected and quantitated by flow cytometry. We also found a dose-dependent apoptotic phenomenon in several selected compounds. The method described here can be used to follow changes in apoptotic cells and it can also be used to count the percentage of apoptotic cells in a culture. Many anticancer drugs cause cytotoxicity by causing cells to undergo apoptosis in aberrant cell cycle regulation, especially in the G_0/G_1 phase and G_2/M arrest phase. That prevents the cellular population from progressing into the S phase of DNA replication after drug treatment.[22,45] In addition, we demonstrated that the cell cycle was slightly inhibited from arresting in the G_0/G_1 phase, and obviously inhibited in the G_2/M phase. The different mechanisms for inducing apoptosis in various cells were analyzed, and the expression of other related family members may be helpful in finding in new approaches to cancer treatment and prevention. The apoptosis inhibition actually resulted in the proliferation of the surviving cells and, notably, although the surviving cells showed more aberrant morphology such as variation in nuclear size, nuclear fragments, and multinucleated cells, all occurred in a dose-dependent manner. Cell cycle analysis after 24 h of compound **2h** treatment showed a significant decrease in S phase cells, with a concurrent increase in G_0/G_1 phase cells, thus suggesting that **2h** induces G_1 arrest. In summary, our studies demonstrated in Jurkat T cells that the cytotoxicity and dose-dependent mechanism of action of anthracene derivatives and mitoxantrone induced a nontypical apoptosis-like pathway. This process may be important for the mechanism of action of anthracenes and further efforts to explore their development appear warranted.

Acknowledgments The present study was supported by National Research Institute of Chinese Medicine Grant NRICM-9206. The authors are also indebted to Dr. K. K. Mayer (Universität Regensburg) for analytical determination for analogues of synthetic compounds.

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